Abnormal miR-214/A20 expression might play a role in T cell activation in patients with aplastic anemia

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Abstract

Aberrant T cell activation is a major cause of aplastic anemia (AA) pathogenesis. Recent studies have shown that miRNAs regulate T cell activation and are involved in AA. A previous study found that miR-214 was significantly up-regulated upon T cell activation in a CD28-dependent fashion by targeting PTEN. However, the expression characteristics of miR-214 and its target genes in AA have not been defined. In this study, target genes for miR-214 were predicted and confirmed by bioinformatics and luciferase reporter assays. The expression levels of miR-214 and target genes were detected in 36 healthy individuals and 35 patients with AA in peripheral blood mononuclear cells by real-time quantitative reverse transcriptase-polymerase chain reaction. Bioinformatics and luciferase reporter assays identified that miR-214 could bind to the A20 3′ untranslated regions. Significantly increased miR-214 and the decreased A20 expression level were detected in the AA patients compared with the healthy group. In addition, significantly increased miR-214 was found in non-severe aplastic anemia compared with severe aplastic anemia patients. These results suggested that the A20 gene was a potential target of miR-214, and elevated miR-214 might medicate T cell activation at least in part by regulating A20 expression in AA. We firstly confirmed that miR-214 regulated A20 expression, and aberrant miR-214/A20 expression might contribute to immunopathology in AA. The miR-214 expression might be used as a potential biomarker that assisted in diagnosing AA severity.

Keywords: A20, Aplastic anemia, miR-214, T cell

1. INTRODUCTION

Aplastic anemia (AA) is an autoimmune disease that is characterized by pancytopenia and bone marrow failure.[1–3] AA is graded primarily on peripheral blood count values into the severe aplastic anemia (SAA), very severe aplastic anemia (VSAA), and non-severe aplastic anemia (NSAA).[4–5] Activated T cells have been considered to be responsible for the pathogenesis of AA.[6] Our previous study has shown that there were an aberrant expression level of TCR signaling pathway related molecules, including CD3ζ, CD28, CTLA-4, and Cbl-b.[7,8] The expression characteristics of these aberrant TCR signaling molecules might partially explain the abnormal T cell activation in AA. However, the precise mechanisms of T cell activation in AA remain unclear.

MicroRNAs (miRNAs) are endogenous small, conserved non-coding RNA molecules that act as posttranscriptional regulators

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Author contributions: Bo Li and Yuping Zhang contributed to the concept development and study design. Qi Shen and Zhi Yu collected AA samples, analyzed clinical data. Chen Lin participated in partial study design. Yankai Xiao, Lixing Guo and Guixuan Huang performed the laboratory studies. Guangxiao Tan, Weifeng Luo, Ming Zhou and Yummiao Li were responsible for collecting healthy individuals' samples and clinical data. Bo Li, Yankai Xiao, Cunte Chen and Xiaohui Chen helped draft the manuscript. All authors read and approved the final manuscript.

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by hybridizing to complementary sequences in 3′ untranslated regions (3′-UTR) of corresponding mRNAs.[9–11] Studies in autoimmune diseases and hematological malignances have demonstrated miRNAs play an important role in regulating gene expression.[12,13] Recent studies have shown miRNAs that regulate T cell activation are involved in AA. For example, downregulation of miRNA-145-5p and miRNA-126-3p has been found in $CD4^+$ and $CD8^+$ T cells, which contributed to aberrant T cell activation by targeting MYC/PI3KR2 in AA patients,[14] and miRNA-34a/DGKζ dysregulation enhanced T cell activation in AA.[15] Nevertheless, these limited data did not completely explain the miRNA-mediated aberrant T cell activation in AA. A recent study has found that co-stimulation by CD28-dependent miR-214 up-regulation promoted T cell activation by targeting the negative regulator PTEN.[16] However, the expression characteristics of miR-214 and its target genes in AA have not been defined. Thus, in this study, we investigated the miR-214 expression level in AA and predicted and confirmed miR-214 target genes by using bioinformatics and luciferase reporter assays to comprehensively understand abnormal T cell activation in AA.

2. MATERIALS AND METHODS

2.1. Cell cultures and clinical samples

Human embryonic kidney (HEK293T) cells were maintained in GlutaMAX High Glucose DMEM medium (Gibco, USA) supplemented with 10% Fetal Bovine Serum (Gibco, USA) and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin). The HEK293T cells were incubated at 37°C in humidified atmosphere of 5% CO_2.

The AA group consisted of 35 patients with newly diagnosed and untreated. There were 17 cases with SAA and 18 cases with NSAA, including 16 males and 19 females (median age: 33 years, range: 9–72 years). The clinical information of the patients was described in Table 1. Thirty-six healthy individuals (16 males and 20 females; median age: 30.5 years, range: 12–62 years) served as a control group (HI). AA diagnosis was established by bone marrow biopsy and peripheral blood counts while excluded with other hematological, autoimmune diseases or infections that caused cytopenia. Healthy individuals and AA patients have signed an informed consent form and this study was approved by the Ethics committee of School of Medicine of Jinan University in accordance to the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) isolation, RNA and DNA extraction, and cDNA synthesis were performed according to the manufacturer's instructions.

Table 1

The demographic and clinical characteristics of AA patients.

Patients characteristics (n = 35)	No (%)
Age (y)	Median 33.0 Range 9.0–72.0
Gender	
Male	16 (45.7)
Female	19 (54.3)
Severity of AA	
NSAA	18 (48.6)
SAA	17 (51.4)
Clinical data	
Hb (g/L)	Median 71
ANC (10^9/L)	Median 0.81
PLT (10^9/L)	Median 15.5

ANC = absolute neutrophil count, Hb = haemoglobin, PLT = platelet.

2.2. Target gene prediction and luciferase reporter vector construction

Target genes for miR-214 were predicted and analyzed by TargetScan (http://www.targetscan.org). The 3′-UTR of the *A20* gene (NM_001270508) contains a putative miR-214 binding site located at 603–623 in the *A20* mRNA sequence.

Segments of the *A20* 3′-UTR were synthesized by Invitrogen (Invitrogen, China). The 3′-UTR segment containing the predicted seed region was as follows: 5′-*TCGAG* AACCA TCCAT GGACTGTGAT TCTGA GGCTGCTGAG ACTGA ACAGT *GTTT*-3′ (forward) and 5′-*AAACA* CATGT TCAGT CTCAGCAGCC TCAGA ATCACAGTCC ATGGA TGGTTC-3′ (reverse). Cleavage sites for XhoI and MssI are shown in italics, and the seed regions are underlined. The mutated seed sequence segment was as follows: 5′-*TCGAG* AACCA TCCAT GGACT GTGAT TACGA GGCCATAGAG ACTGA ACATG T*GTTT*-3′ (forward) and 5′-*AAACA* CATGT TCAGT CTCTATGGCC TCGTA ATCAC AGTCC ATGGA TGGTTC-3′ (reverse). The XhoI and MssI cleavage sites are shown in italics, and the mutated bases are underlined. The mutant *A20* 3′-UTR segment contains six substituted nucleotides corresponding to the seed region of miR-214. The segments of the *A20* 3′-UTR (wild type or mutant) were annealed and inserted into the psiCHECK-2 plasmid (Promega, USA), and psiCHECK-2 plasmid constructs containing the wild type and mutant sequences of the *A20* 3′-UTR (CHK2-A20-WT and CHK2-A20-MU, respectively) were verified by DNA sequencing.

2.3. Dual luciferase assays

HEK293T cells were cultured in 12-well plates (2.5×10^5/well) and grew to approximately 80% confluence after 24 hours. The cells were then co-transfected with the CHK2-A20-WT or CHK2-A20-MU plasmids and miR-214 mimics or without miR-214 mimics which was a negative control. Transfection of vectors and miRNA mimics were performed with Lipofectamine 2000 (Invitrogen, US) according to manufacturer's protocol. The miRNA-214 mimics were synthesized by Guangzhou RiboBio (RiboBio, China). The Dual-Glo Luciferase Assay System (Promega, USA) was used to evaluate the relative activity of Firefly and Renilla luciferase 48 hours after transfection according to manufacturer's protocol. The transfections were performed in duplicate and repeated three times.

2.4. Real-time relative quantitative PCR (qRT-PCR) of miR-214

The SYBR green qRT-PCR assay used for miR-214 quantification was performed with the CFX96 Fast real-time PCR system (Bio-Rad, California, USA) using the miScript SYBR Green PCR kit (Qiagen, Duesseldorf, Germany). Each reaction was performed in a final volume of 25 μl containing 2 μl of cDNA, 2.5 μl of miR-214 primer, 2.5 μl of universal primer and 12.5 μl of 2× QuantiTect SYBR Green PCR Master Mix (Qiagen, Duesseldorf, Germany). The amplification profile was as follows: denaturation at 95°C for 15 minutes followed by 40 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 70°C for 30 seconds. The expression levels of the miRNAs were normalized to RNU6B. The primers used for miRNA analysis were purchased from Qiagen. The $2(^{-\Delta}CT) \times 100\%$ method was used to analyze the miR-214 expression level. Each sample was tested twice.

2.5. Real-time relative quantitative PCR for PTEN, A20 genes

The qRT-PCR using the SYBR Green I method was used to examine the *PTEN* and *A20* gene expression levels using cDNA obtained from PBMCs from AA patients and healthy individuals. The primer sequences used for *A20* and *PTEN* gene amplification were listed in Table 2. The β_2M which served as an internal control primer sequences and PCR conditions have been previously described.[17] The qRT-PCR reactions were performed in a total volume of 25 μl containing 1 μl of cDNA, 0.5 μM of each primer pair, and 11.25 μl of 2.5 × RealMasterMix (Tiangen, Beijing). After an initial denaturation at 95°C for 15 min, 40 cycles of the following procedure were performed using an MJ Research DNA Engine Opticon 2 PCR cycler (BIO-RAD, Hercules, CA, USA): 15 s at 95°C and 30 s at 60°C followed by 1 s at 80°C for plate reading. The $2^{-\Delta CT} \times 100\%$ method was used to represent the gene expression of interest relative to the internal control β_2M. Each sample was tested twice.

2.6. Statistical analysis

The Mann-Whitney test was performed to compare the miRNA and mRNA expression levels between the different groups. The two-tailed Student's *t* test was performed to compare relative luciferase activity between the different groups. Statistical analysis was conducted by GraphPad Prism 7 statistic software and $P < .05$ was considered statistically significant.

Table 2

Primer sequences of *A20* and *PTEN* in qRT-PCR.

Primer	Sequence	Accession no
A20-F	5′-CTGGGACCATGGCACAACTC-3′	NM_001270508.1
A20-R	5′-CGGAAGGTTCCATGGGATTC-3′	
PTEN-F	5′-TGCAGTATAG AGCGTGCAGA-3′	NM_000314.6
PTEN-R	5′-TAGCCTCTGGATTTGACGGC-3′	

3. RESULTS

3.1. Significant up-regulation of miR-214 in AA

It has been reported that co-stimulation by the CD28-dependent up-regulation of miR-214 promotes T cell activation by targeting *PTEN*.[16] Interestingly, our previous study has also found increased CD28 expression in T cells from AA patients.[18] However, the expression characteristics of miR-214 and *PTEN* in AA have not been defined. Thus, we first investigated the expression level of miR-214 and *PTEN* in AA.

The result of qRT-PCR revealed that significantly increased miR-214 expression level (median: 0.044%) was found in AA patients compared with healthy individuals (median: 0.002%, $P < .001$; Fig. 1A), and a significantly increased miR-214 expression level (median: 0.120%) was found in NSAA patients

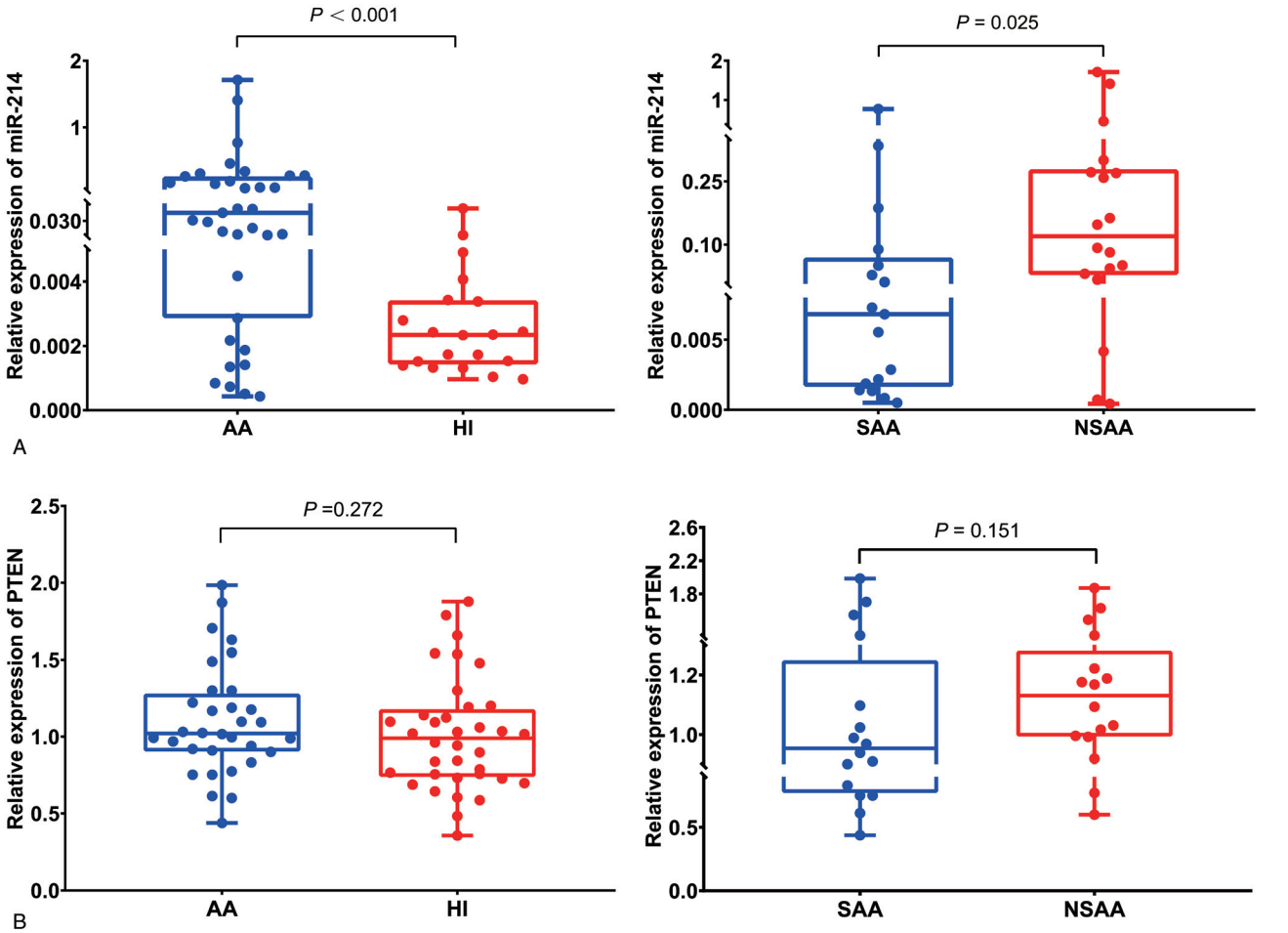

Figure 1. The expression characteristic of miRNA-214 and PTEN in healthy individuals and AA patients. (A) The miRNA-214 expression level in healthy individuals, AA, SAA, and NSAA patients. (B) The PTEN expression level in healthy individuals, AA, SAA, and NSAA patients.

compared with SAA patients (median: 0.007%, $P=.025$; Fig. 1A). However, there was no significant difference in the *PTEN* expression level between patients with AA (median: 1.020%) and healthy individuals (median: 0.989%), and between NSAA (median: 1.131%) and SAA (median: 0.954%) groups as well (Fig. 1B).

3.2. miR-214 binds the A20 3′-UTR and regulates its expression

It is well known that each miRNA has more than one target; thus, we explored whether there were other miR-214 target genes involved in T cell activation in AA. Bioinformatics predictions were used to screen potential genes regulated by miR-214. Using TargetScan (Release 7.1, www.targetscan.org), we obtained a list of genes that were potentially regulated by miR-214. One of these genes, *A20* (also known as *TNFAIP3*), which played a negative regulatory role in T cell activation,[19] was selected for validation.

Based on complementarity of the miR-214 binding sequence, segments of the *A20* 3′-UTR with either the predicted wild type or mutated miR-214 binding site were inserted into the psiCHECK-2 plasmid (Fig. 2A, B). When the luciferase reporter plasmid containing the wild type sequence was co-transfected with miR-214 mimics in HEK293T cells, the luciferase activity of the CHK2-A20-WT vector was observed to decrease by 22.7% compared with the CHK2-A20-MU vector (Fig. 2C). Luciferase activity assays revealed that miR-214 directly bound the *A20* 3′-UTR which leaded to downregulate luciferase activity.

3.3. Significant *A20* down-regulation may be related to abnormal miR-214 regulation in AA

To the best of our knowledge, the expression of *A20* in AA has been rarely reported. Thus, we first investigated the expression level of *A20* in AA. In this study, we found a significantly decreased expression level of *A20* (median: 3.414%) in AA patients compared with healthy individuals (median: 6.059%, $P<.001$, Fig. 3A). However, there was no significant difference in the *A20* expression level between NSAA (median: 3.602%) and SAA (median: 3.408%, Fig. 3A) patients. Our results indicated that up-regulation of miR-214 might have effect on *A20* expression in AA (Fig. 3B)

4. DISCUSSION

It is a common conception that T cells play a key role in the pathogenesis of AA.[20] Abnormal expansion of Th1 and Th17 cells, and a skewed regulatory T cell immunophenotype and function constitute the characteristics of dysregulated immune T cells in AA.[21–23] Moreover, genome-wide transcription analysis has revealed a series of disordered genes in $CD4^+$ and $CD8^+$ T cells from AA patients.[24] T cell activation is important to maintain T cell homeostasis and avoid immunopathology.[25–27] Although the T cell immune pathophysiology of AA is well characterized, the characteristics and mechanisms of abnormal T cell activation in AA remain unclear. More and more data have shown that miRNAs are tightly regulated during T cell activation to enable the establishment of precise T cell immune responses.[28–30] Included in these miRNAs is miR-214, which may be involved in the T cell immune system. A recent study has found that miR-214 is significantly up-regulated upon T cell activation. Moreover, co-stimulatory blockade using CTLA4-Ig resulted in decreased miR-214 expression in alloreactive T cells, suggesting that miRNA-214 is up-regulated in T cells upon encountering antigens in a CD28-dependent fashion due to the direct binding of miRNA-214 with the 3′-UTR of *PTEN*.[16]

In our study, significantly increased miR-214 expression level was detected in AA. But, the expression level of *PTEN* had no

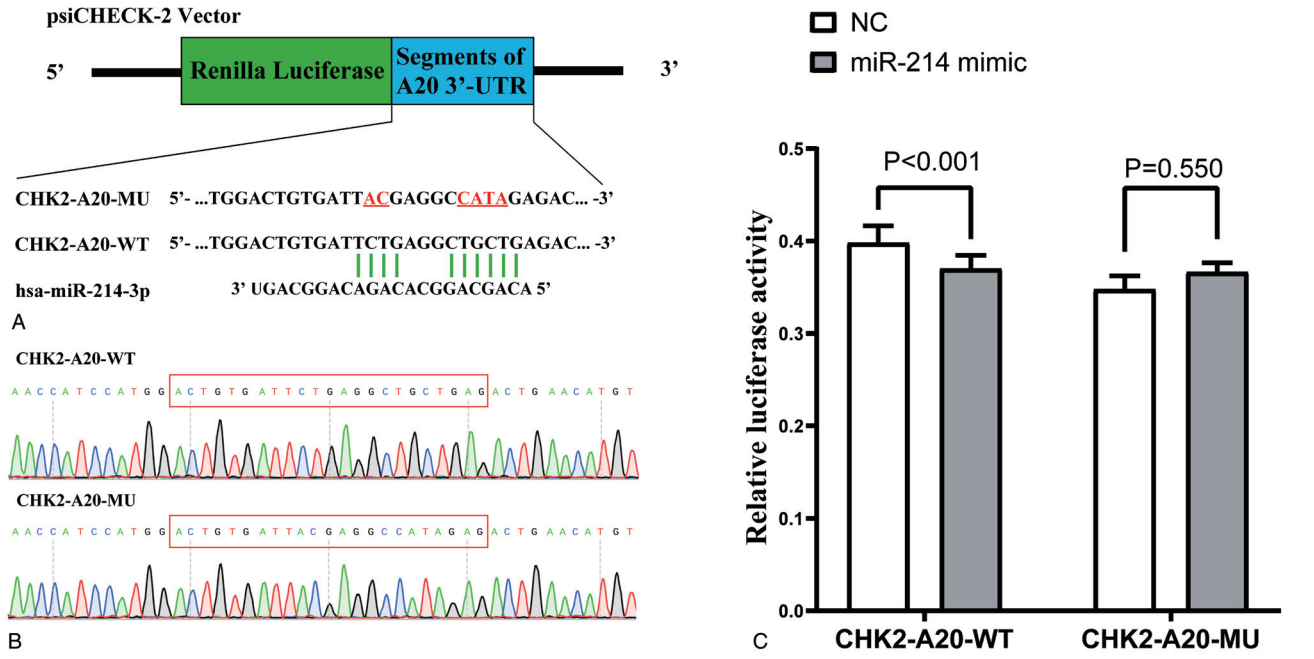

Figure 2. Identification of *A20* as a miRNA-214 target. (A) Wild type and mutant *A20* 3′-UTR segments were cloned into the psiCHECK plasmid. The underlined red letters indicate the *A20* 3′-UTR mutation sites. (B) Wild type and mutant *A20* 3′-UTR segments. The red box indicates the wild type and mutant seed regions. (C) HEK293T cells were co-transfected with CHK2-*A20*-WT or CHK2-*A20*-MU plasmids and miR-214 mimics or without miR-214 mimics. The relative luciferase activity was significantly decreased in CHK2-*A20*-WT cells after 48 h.

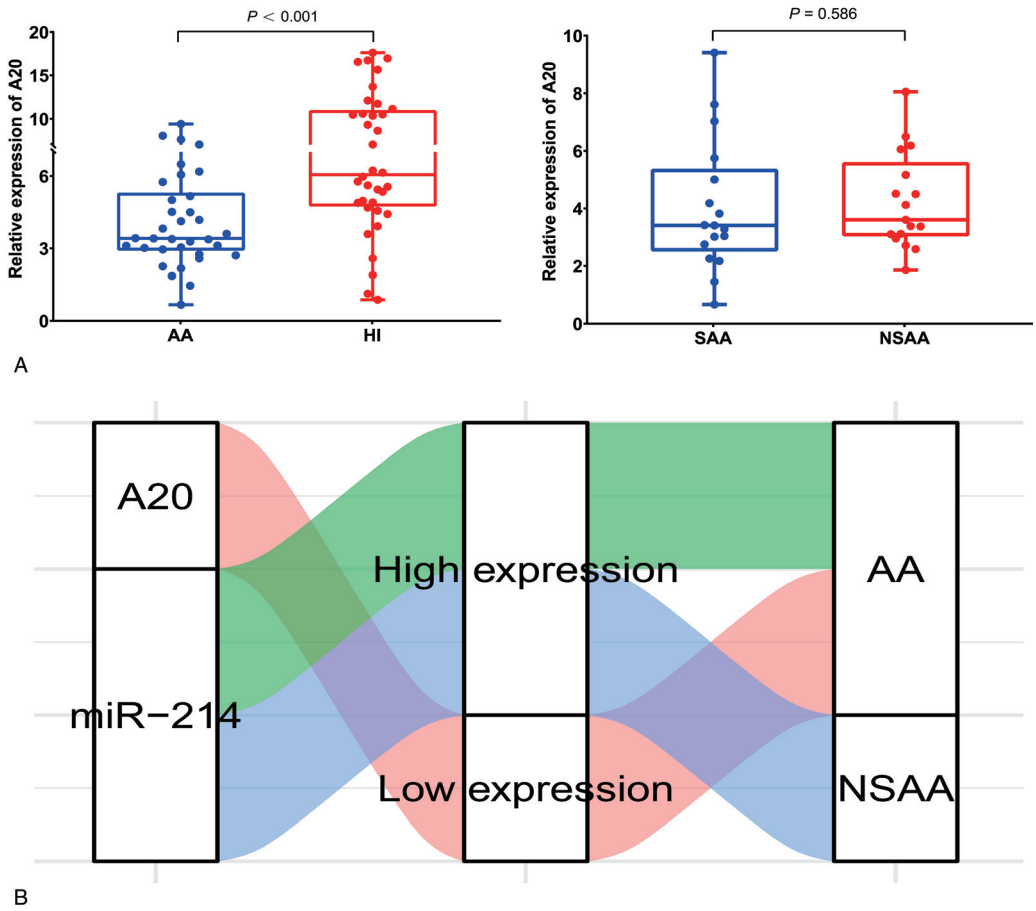

Figure 3. The characteristic of A20, miR-214, and PTEN in AA patients. (A) Decreased A20 expression level in AA patients. (B) The schematic diagram of the expression level of A20 and miR-214 in AA patients and healthy individuals.

significant difference between AA and healthy individuals. Specific miRNA regulation target gene expression is highly dependent on various pathophysiological status. These results might indicate that up-regulation was not directly associated with *PTEN* expression in AA. Hence, further investigation will be needed in a larger cohort of samples to analyze miR-214 whether regulation *PTEN* in AA.

So far, the mechanisms of upregulation of miR-214 in AA have still unknown, we speculated that other miR-214 target genes involved in T cell activation might be regulated by miR-214 in AA. Bioinformatics and luciferase reporter assays identified that miR-214 could bind to the A20 3′-UTR.

A20 was identified as a negative effector of TCR-induced NF-κB activation in autoimmune disease. Dysregulation of A20 has been observed in some autoimmune diseases.[31] For example, *A20* expression was decreased in systemic lupus erythematosus (SLE) patients, and the expression of *A20* appeared to be negatively correlated with the SLE disease activity index and erythrocyte sedimentation rate.[32]

In our study, increased miR-214 and decreased A20 expression characteristics were found in AA; furthermore, luciferase activity indicated that A20 3′-UTR containing the predicted miR-214 binding sequence. The results indicated that up-regulation of miR-214 might have effect on A20 expression in AA.

However, there is one point that needs to be underlined. There are wide individual differences in the miR-214 expression level in AA, and there is a relatively shared *A20* expression level in AA. The expression pattern differences of miR-214 and *A20* suggest that elevated miR-214 may mediate T cell activation in part by regulating *A20* expression in AA. The low *A20* expression level in AA patients may provide a novel molecular mechanism which? the loss of negative regulation of T cell activation plays a crucial role in AA.

The variation in miR-214 expression between NSAA and SAA patients suggests that the function of miR-214 in AA is complex and requires further discovery and verification of other miR-214 target genes that may be involved in immunopathology in AA. We previously found a more significantly increased CD3ζ mRNA expression in NSAA.[4] The results of present studies might evidence that aberrant T cell activation is distinct in different severities of AA once again. Therefore, it is necessary to clarify the specific changes and mechanism among different severities of AA.

5. CONCLUSIONS

We for the first time analyzed the expression characteristics of miR-214 and *A20* in AA patients and confirmed that miR-214 directly regulates A20 expression. These findings suggested that aberrant miR-214 and *A20* expression might contribute to immunopathology in AA, and miR-214 also might be used as a potential biomarker that assisted in diagnosing AA severity.

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